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EFFECT OF HEPATOTOXIC NONADECAFLUORODECANOIC ACID ON GENE EXPRESSION IN RAT LIVER

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Nonadecafluorodecanoic acid (NDFDA), a biological test model for similar compounds used to produce aqueous film-forming foams, is a potent hepatotoxin. Since the compound has been shown to modify cellular membrane systems, the effect of NDFDA on membrane-dependent functions of genetic expression has been investigated. Messenger RNA transport through nuclear pores in a cell-free system derived from rat liver, was significantly inhibited when the nuclei were derived from NDFDA-treated rats. The induction of the hepatic enzyme tyrosine aminotransferase by hydrocortisone and insulin was near normal. In contrast,

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Block 20 - Abstract (Continued)

pretreatment with NDFDA inhibited induction by glucagon, suggesting that some receptor or post-receptor step in the glucagon-mediated activation of the membrane-bound adenylate cyclase system was defective. Surprisingly, NDFDA did not significantly affect the binding of polyribosomes to the endoplasmic reticulum membrane system. Analysis of the proteins from liver cytosol and blood plasma by sodium dodecyl sulfate- polyacrylamide gel electrophoresis indicated significant changes in the relative concentration of specific proteins. It is concluded that NDFDA has specific effects on genetic expression in rat liver.

The experiments reported herein were conducted according to the principles described in "Guide for the Care and Use of Laboratory Animals" prepared for the Committee on Care and Use of Laboratory Animals, DHHS Publication No. (NIH) 78-23, revised 1982.

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Research Objectives

Preamble:

Nonadecafluorodecanoic acid (NDFDA) is extremely toxic and may serve as a model compound for evaluating the health hazard associated with use of polyfluorinated chemicals as film forming foam fire extinguishants or in rendering porous materials oil and water resistant. One necessary aspect of evaluating the health hazard of NDFDA is to elucidate the mechanism of its toxicity. Particularly appropriate is an evaluation which will permit the development of appropriate diagnostic and therapeutic methodology for short duration-high level infrequent exposure. Earlier studies at AFAMRL, Wright Patterson AFB (cf 1) had strongly suggested that NDFDA caused changes in plasma membranes. In particular, NDFDA appeared to be a hepatotoxin, although other organs (bone marrow, thymus, stomach and testes) were also affected. The toxicity of the drug is similar to that of 2,3,7,8-tetrachloro-dibenzodioxin (TCDD), as shown by recent studies at AFAMRL (2).

As SCEE Fellow at AFAMRL, WPAFB, in 1982, this investigator studied the effect of NDFDA treatment on rat liver stearoyl CoA-desaturase and associated electron transport functions in the microsome fraction. These studies (3) suggested that NDFDA caused a reduced nutritional inducibility of stearoyl-CoA desaturase, a marked decrease in the rate of microsomal electron transport from NADH through cytochrome b_5 to terminal oxidases (including the desaturase) and to molecular oxygen and an increase in the concentration of cytochrome P-450. It was concluded from these and other results that the unique effects of NDFDA on liver reflects a direct or indirect modulation of genetic expression and/or specific changes in membrane systems, rather than non-specific cellular damage.

Objectives of Present Study:

The overall objective was to assess the effect of NDFDA on those aspects of regulation which may be affected by toxin-induced changes in the membrane, or which if defective could promote changes in membrane structure and function. The specific objectives of this study were as follows:

(i) To evaluate the responsiveness of hepatocytes in the liver of NDFDA-treated rats to hormones whose action is (eg. insulin, glucagon), or is not (eg. hydrocortisone) mediated by membrane receptors. Tyrosine aminotransferase induction was selected since this hepatic enzyme is induced by each of these 3 hormones, and in one case this induction involves the interaction of a hormone receptor and the adenylate cyclase system.

(ii) To determine the extent to which the modification of the cellular membrane systems by NDFDA affects the functional competence of the hepatocyte nucleus for RNA processing and transport. This lab has developed a reconstituted cell-free system consisting of isolated nuclei in surrogate cytoplasm which supports RNA processing and transport. The system supports the processing and transport of both ribosomal and messenger RNA as the corresponding ribonucleoproteins.

(iii) To evaluate the effect of NDFDA on the association of cytoplasmic polyribosomes with the endoplasmic reticulum. Normally approximately 2/3 of the cytoplasmic polyribosomes are associated with the intracellular membrane system known as the rough endoplasmic reticulum. The remainder are membrane-free.

(iv) To compare the soluble liver cytoplasmic protein and blood plasma profile of normal and NDFDA treated rats by polyacrylamide gel electrophoresis. This analysis will indicate whether NDFDA causes changes in the relative concentration of proteins in these cellular compartments/body fluids.

(v) To assess the effect of NDFDA on cellular (particulary tumor) growth. (This aim replaced the assessment of NDFDA on apolipoprotein synthesis which proved to be impractical).

(vi) To make an assessment whether detoxification of NDFDA occurs via the glucuronidation pathway. This new aim arose from other ongoing studies on anti-carcinogens in this lab which indicated that this is an important pathway for detoxification of toxic carcinogens. A failure of enhancing the efficiency of this pathway to reduce NDFDA toxicity might indicate that the extreme prolonged toxicity is due in part to inability of detoxification mechanisms to deal with the compound and to assist in clearing it from the body in general, and membrane systems in particular.

Status of Research

The following results were obtained during the 9 month research period of this AFOSR Minigrant.

(i) Treatment with NDFDA

The experiments reported below (with exception of those on tumor-bearing rats) were carried out on 200 gm rats of the Fischer strain maintained on rat chow. Since NDFDA curtails food intake, untreated pair-fed rats served as appropriate controls. Stock NDFDA (Aldrich Chem. Co.) is prepared in propylene glycol:water (1:1,v/v) to give a concentration of 25 mg/ml.

In all of the experiments reported below, the animals were treated with NDFDA at the LD_{50} of 50 mg/kg, at the days post-treatment indicated. The comparisons are primarily between NDFDA-treated rats and pair-fed controls. Normal controls are sometimes included to evaluate the effect of food restriction, since the NDFDA-treated animals are anorexic (2).

(ii) Effect of NDFDA on the induction of hepatic tyrosine aminotransferase

Optimal inducing doses of hydrocortisone sodium succinate (40 mg/kg body weight), insulin (0.75 units/100 gm) and glucagon (1.5 mg/kg) were injected intraperitonially (4,5). NDFDA-treated and control rats were tested for the hormonal inductions of hepatic tyrosine aminotransferase 5 days after NDFDA (or vehicle) treatment. The livers were removed under ether anesthesia for processing at the times after hormone administration indicated in the table; these times correspond to the time of maximum induction by each of the hormones. The tyrosine transaminase activity in the liver cytosol was determined spectrophotometrically as previously described (4,5). The activity is expressed as micromoles of p-hydroxyphenyl pyruvic acid produced per hour from tyrosine.

Table 1. Effect of NDFDA on the Induction of Hepatic Tyrosine-Aminotransferase Activity

Treatment (Inducing agent)	Hours post-treatment*	Tyrosine Aminotransferase (μMoles product/hr) Controls	NDFDA-Treated
Saline (control)	2	9.42 ± 0.3	18.22 ± 1.3
Saline (control)	3	9.51 ± 0.4	18.15 ± 1.3
Hydrocortisone	4.5	28.16 ± 1.4	30.36 ± 1.0
Insulin	3	20.06 ± 1.0	32.38 ± 1.8
Glucagon	2	23.74 ± 1.0	17.11 ± 1.3

*Times shown are times of maximum induction for each of the hormones.

The data in Table 1 indicate that the enzyme is partially (2-fold) induced in NDFDA treated rats, due probably to high circulating levels of corticosterone, released in response to toxicity-related stress. Hydrocortisone further induces the enzyme 1-2 fold in both the pair-fed controls and the NDFDA-treated rats. Insulin also induces the enzyme in both the control and experimental rats. However, glucagon induces the enzyme in the control, but not in the NDFDA-treated animals. Since in contrast to induction by hydrocortisone and insulin, induction by glucagon involves the activation of a membrane-bound

adenylate cyclase by a membrane-bound glucagon receptor in close proximity. This activation occurs upon binding of the glucagon to the receptor. Therefore, decreased induction by glucagon in NDFDA-treated rats may reflect NDFDA-induced changes in the plasma membrane. Furthermore, the non-responsiveness of the adenylate cyclase system to one regulator, may indicate that it is also non-responsive to other regulators.

(ii) Effect of NDFDA on RNA transport, a nuclear membrane function.

Messenger and ribosomal RNA are transported from the nucleus to the cytoplasm via nuclear pores embedded in a bilayer lipoprotein nuclear membrane (6). There is some evidence (6,7) that nutritional or temperature-induced changes in the nuclear membrane modifies (reduces) transport through the pores, possibly by changing the size or conformation of the pores. To test the effect of NDFDA pretreatment, the nuclear RNA of NDFDA treated and pair-fed rats was prelabeled with ^{14}C-orotate *in vivo* for 30 min, the liver nuclei was isolated, then incubated in a surrogate cytoplasm containing 15 mg/ml cytosol protein, ATP, an ATP-regenerating system, salts, nuclear stabilizers and ribonuclease inhibitors (9-11). This system transports consecutively labeled tRNA, messenger RNA and ribosomal RNA in three phases consistent with their *in vivo* pool sizes and processing times (9). Thus, during a 30 min *in vitro* incubation at 30°C, only labeled t-RNA (10-15%) and labeled messenger RNA (>80%) are transported from 30 min prelabeled nuclei (9). The transport curve is approximately hyperbolic. This release is dependent not only on energy (eg. ATP), but also on a 35 Kd phosphoprotein in the cytosol (11). Since any effects due to NDFDA may be due to changes in activity of the cytosol factor, or to the nuclear transport system, cross-over experiments, involving the incubation of nuclei in homologous and heterologous cytosol is carried out to localize the defect.

Shown in Table 2 is labeled RNA transport from 30 min prelabeled nuclei during a 30 min *in vitro* incubation in medium containing homologous or heterologous cytosol. Both nuclei and cytosols were derived from the liver of control or NDFDA-treated rats 5 days after treatment. The extent of transport of the labeled RNA, which is mainly messenger RNA, is expressed as % nuclear cpm transported as RNA.

Table 2. Effect of NDFDA Pretreatment of Liver Donors on RNA Transport in a Liver-derived Cell-free System.

Pretreatment* of Rats Donating: Liver Nuclei	Liver Cytosol	% Nuclear cpm Transported at 30 min.
None (control)	None (control)	6.66
None (control)	NDFDA	5.63
NDFDA	None (control)	3.46
NDFDA	NDFDA	2.94

*Rats were tested 5 days after receiving 50 mg/kg of NDFDA or vehicle (controls).

The data in Table 2 demonstrate that pretreatment of rats with NDFDA does reduce by approximately 50% RNA transport in cell-free systems derived from their livers. Furthermore, the crossover experiments indicate that this reduction is due primarily to changes in the nuclei. Whether this significant decrease is due, as we suspect, to changes in the nuclear membrane awaits further study. This modification of genetic expression at the post-transcriptional level could account for some changes in the concentration of cytoplasmic and plasma proteins noted below.

(iii) Effect of NDFDA on the association (binding) of cytoplasmic polyribosomes with the endoplasmic reticulum membrane system in rat liver cytoplasm.

If the NDFDA affects plasma membranes and nuclear membranes it may also affect, or modify, other intracellular membrane systems such as the mitochondrial membrane or the rough and smooth endoplasmic reticulum. In the present investigation we have investigated the effect of pretreatment of rats with NDFDA on the binding of the polyribosomes to the endoplasmic reticulum to form the rough endoplasmic reticulum. In rat liver, approximately 60-65% of the polyribosomes are normally bound and are thought to synthesize proteins for export (eg. albumin and other proteins) while 30% are membrane-free and are thought to be involved in synthesis of proteins involved in the "house-keeping" functions of the cell (cf. 12).

The proportion of free and bound polyribosomes was estimated in liver homogenates of NDFDA-treated and pair-fed rats 5 days post-treatment. Following an initial separation of the rough endoplasmic reticulum with bound polyribosomes from the pelleted free polyribosomes on a 0.5 M/2.0M discontinuous sucrose gradient (14 hrs at 105,000 g) both fractions were treated with 1.25% deoxycholate to release "bound" from the membranes, and to purify the "free" from absorbed contaminating proteins. The pure polyribosomes from both fractions were isolated in pure form by recentrifuging the detergent-treated fractions through a discontinuous 0.5M/1.5M sucrose gradient at 105,000g x 8 hrs. The polyribosomal RNA was quantitated in aliquots of the free and bound fractions using the relation 1.0 A_{260} unit = 50 mg of RNA and the results are summarized in Table 3. In this experiment the rats were sacrificed 5 days after NDFDA treatment or initiation of pair-feeding.

Table 3. Effect of NDFDA on binding of polyribosomes to liver endoplasmic reticulum.

Pretreatment of liver donors	Ratio (±S.E.) Free-Bound Polyribosomes*
None (controls)	32 ± 1
NDFDA	36 ± 5

*Correction applied for recovery of bound polyribosomes (Ref. 11).

Surpisingly there was little difference in the extent of binding of polyribosomes to the endoplasmic reticulum. Since only gross differences would be detected by this procedure, these results suggest, but do not prove, that the synthesis and export of proteins to the blood from the liver is completely normal in NDFDA-treated rats.

(iv) Effect of NDFDA on the liver cytosol and plasma protein profiles

The liver cytosol protein profile reflects the synthesis and steady state level of proteins involved in some of the so-called "house-keeping functions" of the liver. The plasma protein profile reflects primarily the accumulation/steady state level of proteins exported from the liver and from lymphocytes, although lesser amounts of other proteins may have their origin from other tissues. In the present series of experiments the liver cytosol and blood plasma was obtained from rats 5 days after NDFDA or initiation of pair feeding. The liver cytosol represents the 105,000 g supernatant of a liver homogenate while blood plasma was prepared by removing cellular elements from heparinized blood; the blood was obtained from rats by cardiac puncture under light ether anaesthesia. Equivalent aliquots of protein from NDFDA-treated and

untreated rats were subjected to one dimensional sodium dodcyl sulfate-polyacrylamide gel electrophoresis, which separates the polypeptides according to molecular weight (9-1). Following electrophoresis the gels were stained with Coomassie Brilliant Blue then directly photographed and subjected to densitometric analysis with white light in a Beckman DU-8 spectrophotometer equipped with a slab gel scanning system. The results are sumamrized in the photographs of Fig 1(a) and 1(b) and the densitometric scans of the gels shown in Fig 2(a) and (b).

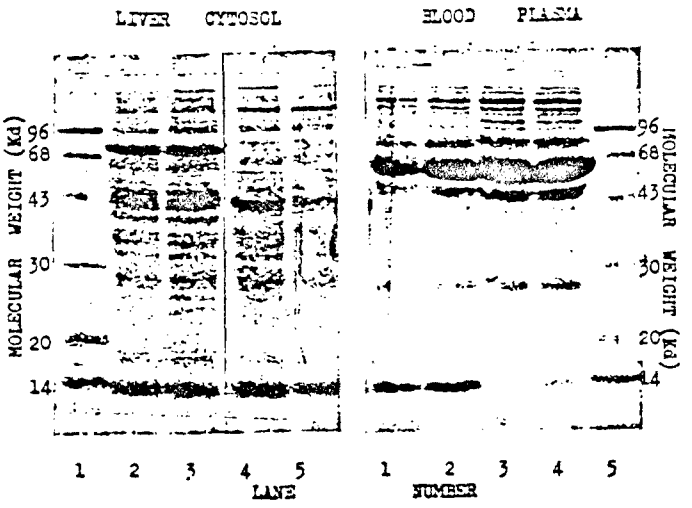

Fig. 1. Protein profiles of liver cytosol and blood plasma of NDFDA-treated and pair-fed control rats. (a) Liver cytosol: Lane 1, protein standards; Lane 2,3, liver cytosols from NDFDA-treated rats; Lanes 4,5, liver cytosols from pair-fed controls. (b) Blood plasma: Lane 1,2, plasmas from NDFDA-treated rats; Lanes 3,4, plasmas from pair-fed controls; Lane 5, protein standards. In (a) and (b) each sample lane represents a different rat.

The data in Figs. 1 and 2 indicate that NDFDA induces some major changes in the protein profiles of both liver cytosol and blood plasma. In this experiment, the cytosol and plasma was analyzed 5 days after NDFDA treatment or the initiation of pair-feedings in the controls. In particular, the pattern in the liver cytosol from NDFDA-treated rats in characterized by a high concentration of a protein with a molecular weight slightly greater than that of serum albumin which has a molecular weight of 68,000 daltons. Other significant differences occur in the region of 40 to 50 Kd. A component with a molecular weight of slightly less than 14 Kd also appears to be at a higher concentration in the liver cytosol from NDFDA-treated rats. Differences are also observed in the relative concentration of proteins in the plasma of NDFDA-treated and control rats. Most significant is the presence of a protein in the plasma of NDFDA-treated rats with a molecular weight of approximately 18,000 daltons and which is in very low concentration in the plasma of pair-fed control rats. Differences in the relative concentration of proteins are also seen in the region of 30-35 Kd, and at approximately 25 Kd.

(v) Toxic effect of NDFDA on a dimethyl-benzanthracene-induced primary mammary tumor in the rat.

The toxicity of NDFDA to normal tissues is well documented in the rat (1,2). It is characterized by weight loss, damage to specific organs including the liver and an increase in the ratio of liver:body weight. As indicated by the data in Table 4, 40 mg/kg of NDFDA appears to induce a decrease in the size of mammary tumors, induced in female Sprague Dawley rats by dimethylbenzanthracene as previously described (13). In these experiments, the

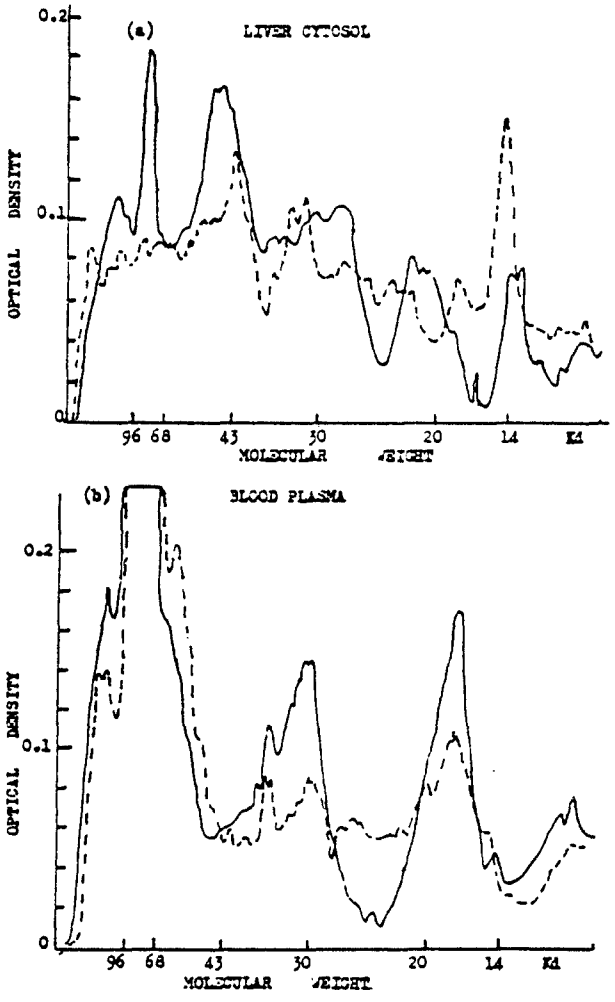

Fig. 2. Densitometric tracings of Coomassie Brilliant Blue stained electrophoretogram of (a) liver cytosol and (b) blood plasma proteins. The protein profiles of both NDFDA-treated (——) and pair-fed control (----) rats are shown.

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tumors were measured at regular intervals by use of vernier calipers and tumor volume was calculated form the relation "length" (longest dimension) + width (narrowest dimension) in cm (cf 13).

Table 4. Effect of NDFDA on the growth of DMBA-induced rat mammary tumors.

	Pretreatment of tumor-bearing rats	Rat number	Number of tumors	Change in tumor volume (l + w in cm)
1.	None (controls)	1	2	+2.6, 0.0
		2	3	+0.6, +2.4, +1.2
		3	4	+1.2, +0.8, +0.9, -1.2
		4	1	+1.2
		5	1	+2.2
2.	NDFDA	1	3	-3.2*, -2.5*, -0.2
		2	1	-0.2
		3	3	-1.8*, -1.6*, -1.4*
		4	3	-2.8, -0.8, -2.4*
		5	4	-2.9, -2.8, -2.0*, -2.3*

*Tumors disappeared completely.

(vi) Effect of an inhibitor of deglucuronidation on the toxicity of NDFDA.

One of the major pathways of detoxification is via glucuronidation of hydroxylated intermediates. The extent of conversion of a toxic substance to the corresponding glucuronide is determined by both the rate of glucuronidation and deglucuronidation. The latter component is of particular interest, since the enzyme catallyzing de-glucuronidation i.e. β-glucuronidase, can be specifically inhibited with 2,5-di-O-acetyl-D-glucaro-1,4:6,3-dilactone (DAGDL), a slow release form of 2,4 glucurolactone (the specific inhibitor) when given by mouth (14). DAGDL has been shown to decrease the effective concentration of carcinogens in vivo when given just prior to the carcinogens (14). Furthermore, there is evidence that carboxylic acids can undergo glucuronidation after metabolism in vivo (15). There was the possibility that metabolic products of NDFDA were also subject to glucuronidation and that its toxicity could be decreased by pre-treatment with DAGDL. The results of an experiment designed to test this possiblity is summarized in Table 5. In this experiment 400 mg/kg of DAGDL was administered to the rats by stomach tube 0.5 hrs before administration of NDFDA, with a second dose of DAGDL 2.5 hrs after administration of NDFDA. The results indicate that DAGDL has no effect on NDFDA-induced toxicity.

Table 5. Effect of DAGDL treatment on NDFDA-induced weight loss.

Treatment		Rat	Net change in
NDFDA	DAGDL	Number	body weight at 7 days
+	-	1	-68 gm
+	-	2	-60 gm
+	+	3	-66 gm
+	+	4	-84 gm

Note: The ratio of liver:body weight in both NDFDA and NDFDA + DAGDL-treated rats was approximately 0.09 as compared to 0.03 in normal rats.

CONCLUSIONS

The present results are consistent with at least part of the NDFDA induced toxicity being associated with membrane effects. Regulation of adenylate cyclase in the plasma membrane may be modified in NDFDA-treated rats and this possibility warrants further investigation. Such a defect could effect a number of cellular functions through a cascade effect. Similarly NDFDA appears to

modify RNA transport through the nuclear pores. Although NDFDA does not have drastic effects on the binding of polyribosomes to the endoplasmic reticulum, this analysis is subject to approximately a 10-15% error. This is an important point since one of the bands which is particularly prominant in the in the cytosolic profile of NDFDA-treated rats has a molecular weight slightly greater than that of albumin and could represent pre-albumin or some other protein of comparable molecular weight which is normally transported from the liver. NDFDA shows significant anti-tumor activity in the case of the rat mammary tumor system. As an anti-cancer agent, NDFDA itself would be impractical because of its severe toxicity to the host. Attempts to reduce toxicity by enhancing the extent of glucuronidation were unsuccessful, although a similar approach was successful with a range of carcinogens of varying structure. This suggests that NDFDA may owe its extreme prolonged toxicity, in part, to the lack of an effective detoxification system for this compound.

(d) Publications:

Webb, T.E. Modification of Genetic Expression in the rat by Nonadecafluorodecanoic Acid, in preparation for publication in Arch. Toxicology or similar journal. (Copy of manuscript will be sent to AFOSR.)

(e) Personnel:

Webb, T.E. carried out most of the research.
French, B.T. provided part-time technical assistance.
Walaszek, M. provided help with technical problems.

(f) Interactions:

Consultive function: At the request of Cols. (Drs.) Andersen and Olsen and Ms. George at AFAMRL we have provided consultation and technical help to Sgt. J.D. Robinson at AFAMRL on the purification of α_{2u}-globulin from male rat urine and have purified some of this protein for them. The purification and development of an immunoassay for this protein are related to the ongoing studies on NDFDA toxicity at AFAMRL (Toxicology), WPAFB.

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